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Analysis of *Claviceps africana* and *C. sorghi* from India using AFLPs, EF-1α gene intron 4, and β-tubulin gene intron 3

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ARTICLE INFO

Article history:
Received 18 January 2005
Received in revised form
14 November 2005
Accepted 16 December 2005
Published online 24 March 2006
Corresponding Editor: Jeffrey K. Stone

Keywords:
Clavicipitaceae
Coevolution
Ergot
Molecular phylogeny
Sorghum

ABSTRACT

Isolates of *Claviceps* causing ergot on sorghum in India were analysed by AFLP analysis, and by analysis of DNA sequences of the EF-1α gene intron 4 and β-tubulin gene intron 3 region. Of 89 isolates assayed from six states in India, four were determined to be *C. sorghi*, and the rest *C. africana*. A relatively low level of genetic diversity was observed within the Indian *C. africana* population. No evidence of genetic exchange between *C. africana* and *C. sorghi* was observed in either AFLP or DNA sequence analysis. Phylogenetic analysis was conducted using DNA sequences from 14 different *Claviceps* species. A multigene phylogeny based on the EF-1α gene intron 4, the β-tubulin gene intron 3 region, and rDNA showed that *C. sorghi* grouped most closely with *C. gigantea* and *C. africana*. Although the *Claviceps* species we analysed were closely related, they colonize hosts that are taxonomically very distinct suggesting that there is no direct coevolution of *Claviceps* with its hosts.

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Introduction

Ergot or sugary disease was first recorded on sorghum (*Sorghum bicolor*) by McRae (1917), who observed the sphacelial stage in the former state of Mysore in India in 1915. In India, losses of 10–80 % due to ergot have been reported in hybrid sorghum seed production fields (Bandyopadhyay *et al.* 1998). Since 1995, sorghum ergot has moved rapidly on a global scale, prompting much concern for worldwide sorghum production (Bandyopadhyay *et al.* 1996, 1998). The disease is now established in parts of South and Central America, Australia, Japan, and the USA (Bandyopadhyay *et al.* 1998). Sorghum ergot is caused by three different *Claviceps* species that are separated based on alkaloid content and morphological characters. The three species are *C. sorghi* (Kulkarni *et al.* 1976) described from India, *C. africana* (Frederickson *et al.* 1991) first described from Zimbabwe and *C. sorghicola* (Tsukiboshi *et al.* 1999) described from Japan. Although *C. africana* has been identified as the species causing sorghum ergot in many new regions of the world since 1995, *C. sorghi* was until recently considered to be the only ergot pathogen in India.

Although *C. sorghicola* differs profoundly in its morphology from both *C. africana* and *C. sorghi*, the latter two species are more difficult to distinguish, especially in the earlier stages

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0953-7562/$ – see front matter Published by Elsevier Ltd on behalf of The British Mycological Society.
doi:10.1016/j.mycres.2005.12.006

of sphacelial development. This similarity led to classification of both anamorthic stages under *Sphacelia sorghi* (McRae 1917). Although the shape and size of conidia is almost indistinguishable in *C. africana* and *C. sorghi*, they differ in the shape of sclerotia (*C. africana* assuming seed shape and hidden in glumes, *C. sorghi* is larger, conical, protruding). In addition, sclerotia of *C. sorghi* germinate readily into perithecial heads, whereas the sexual stage of *C. africana* is very rare (Frederickson *et al.* 1991). During Indian surveys of sorghum ergot in 1914 to 1917 (Ajrekar 1926) and 1946 (Ramakrishnan 1948) in Karnataka and Andhra Pradesh States, and during the early 1960s (Singh 1964) in Akola (Maharashtra) and Dharwad (Karnataka), large elongated sclerotia were observed. However, both types of sclerotia were found in the late 1970s (Kulkarni *et al.* 1976; Sangitrao & Bade 1979) which might have been either due to variability in the size of sclerotia of *C. sorghi*, or to the beginning phase of establishment of *C. africana*.

Since the late 1980s, the typical protruding sclerotia of *C. sorghi* have been rare in Karnataka, Andhra Pradesh and Maharashtra, but in 1997, *C. africana* was unequivocally identified from several locations in India based on its secondary metabolites and rDNA sequence (Bogo & Mantle 1999; Pažoutová *et al.* 2000). *C. africana* has displaced *C. sorghi* and now represents the dominant cause of sorghum ergot in India (Pažoutová & Bogo 2001). The ability of *C. africana* to produce large numbers of secondary conidia relative to *C. sorghi* (Bandyopadhyay *et al.* 2002; Muthusubramanian *et al.* 2006) could be a primary reason for its dominance.

During the 1999–2000 growing seasons, ergot disease surveys were conducted in India and severe disease was recorded in major sorghum growing areas of the states of Andhra Pradesh, Karnataka, Maharashtra, Rajasthan, Uttar Pradesh, Tamil Nadu, and Gujarat (Bandyopadhyay *et al.* 2002; Navi *et al.* 2002). Three particular epidemics were especially severe and led to a total failure of the sorghum grain harvest. In September 1999, a severe epidemic occurred in the Rampur and Moradabad districts of Uttar Pradesh. In October 1999, toward the end of the rainy season, another epidemic occurred in the Machinenapally village of the Mahbubnagar district of Andhra Pradesh, and finally, in September 2000, several other *mandals* (particularly Kalwakurthy *mandal*), adjoining Machinenapally village, were also severely affected. Material was collected from the above states and regions to provide samples for cultural and molecular studies to determine the relative prevalence of *C. africana* and *C. sorghi* in India (Bandyopadhyay *et al.* 2002; Navi *et al.* 2002; Muthusubramanian *et al.* 2006).

Molecular tools applied to studies of *Claviceps* species, which attack sorghum, have been limited (Komolong *et al.* 2002; Partridge *et al.* 2000; Pažoutová 2001; Pažoutová *et al.* 2000; Tooley *et al.* 2000, 2001, 2002) whereas those applied to the most widely studied species, *C. purpurea*, have been more plentiful (Annis & Panaccione 1998; Garre *et al.* 1998; Giesbert *et al.* 1998; Jungehülsing & Tudzynski 1997). Nuclear SSU (Glenn *et al.* 1996; Partridge *et al.* 2000; Spatafora & Blackwell 1993) and LSU (Kuldau *et al.* 1997) rDNA sequences have been used in molecular studies of *Claviceps* species to assess taxonomic relationships and develop identification techniques.

Pažoutová (2001) analysed the genus *Claviceps* using ITS regions of ribosomal DNA and found two highly supported clades; one that included *C. purpurea* and *C. paspali*, and one that included *C. africana* and *C. sorghicola*. Tooley *et al.* (2002) characterized the intron 3 region of the β-tubulin gene, and intron 4 of the translation elongation factor gene to determine phylogenetic relationships among *C. africana*, *C. sorghicola*, *C. purpurea*, *C. fusiformis*, and *C. paspali*, two of which (*C. africana* and *C. sorghicola*) are pathogens of sorghum. For *C. africana*, AFLP analysis performed on isolates from worldwide sources (Tooley *et al.* 2000, 2002) showed that two major groupings existed, one consisting of Australian, Indian, and Japanese isolates and the other of USA, Mexican, and African isolates.

Additional insight into species relationships in the genus *Claviceps* is desirable as they have been so little studied. For species that coexist in the same region and attack sorghum (such as *C. africana* and *C. sorghi*) it would be useful to examine their overall genetic makeup, as well as specific regions of the genome, to determine how divergent they are genetically, and whether the two species may be undergoing genetic exchange. We thus analysed both AFLP diversity, as well as similarity, within two specific regions of the genome (intron 3 of the β-tubulin gene and intron 4 of the translation elongation factor gene EF-1α) for *C. africana* and *C. sorghi* isolates collected in India during the epidemics of 1999–2000 and 2000–2001. The cultural and morphological characteristics of isolates collected during the 1999–2000 epidemics have been characterized in a companion study (Muthusubramanian *et al.* 2006).

Materials and methods

Cultures

During 1999–2000 (October to February), an intensive disease survey was carried out in India in which disease incidence and severity were recorded, infected plant material was collected, and pathogens were cultured from field collections (Muthusubramanian *et al.* 2006). From the infected material collected, 89 single conidial isolates were cultured and their cultural characteristics determined (Bandyopadhyay *et al.* 2002; Muthusubramanian *et al.* 2006; Navi *et al.* 2002). Based on cultural characteristics, isolates were placed into two major groups that corresponded to *Claviceps sorghi* and *C. africana* (Muthusubramanian *et al.* 2006). *C. africana* was recorded in all the sorghum growing regions of Maharashtra, Karnataka, Andhra Pradesh, Tamil Nadu, Uttar Pradesh, Rajasthan and Gujarat, while the *C. sorghi* isolates were restricted to Northern Andhra Pradesh (Adilabad district) and Maharashtra (Yavatmal district). Twenty-one additional isolates were obtained from collections made in the 2000–2001 rainy season, largely in the Mahbubnagar District of Andhra Pradesh (Table 1). We also included in the analysis data for four isolates obtained from material collected in India in 1997 (two from Patancheru, one from Dharwad, and one from Akola) and analysed previously (Tooley *et al.* 2002). Cultures were established by diluting honeydew *ca* 100-fold in sterile distilled water and streaking onto plates of 2 % water agar containing 100 ppm ampicillin and streptomycin sulfate. Plates were incubated at 22 °C in darkness, and individual germinated conidia were transferred to plates of potato dextrose agar (PDA) where they grew into colonies. Cultures used in the study are preserved in the Indian Type Culture Collection (IARI, New Delhi).

Table 1 – Sources of Indian isolates of *Claviceps africana* and *C. sorghi* used in this study[a]

Isolate	State	District	Locality	Cultivar	Date collected
Cls1	Andhra Pradesh	Medak	Patancheru	Unknown	1997
Cls4	Andhra Pradesh	Medak	Patancheru	Unknown	1997
NAP1	Andhra Pradesh	Ranga Reddy	Tammalonigudem	Local	Oct. 99–Feb. 00
NAP 2	Andhra Pradesh	Nalagonda	Chakalisherupally	Yellow Jowar	Oct. 99–Feb. 00
NAP3	Andhra Pradesh	Nalgonda	Injamura	Local	Oct. 99–Feb. 00
NAP5	Andhra Pradesh	Adilabad	Dongregaw	CSH0	Oct. 99–Feb. 00
NAP6	Andhar Pradesh	Adilabad	Gudihatnur	CSH9	Oct. 99–Feb. 00
NAP7	Andhra Pradesh	Adilabad	Sitagondi	CSH9	Oct. 99–Feb. 00
NAP9	Andhra Pradesh	Adilabad	Kamalapuram	CSH9	Oct. 99–Feb. 00
NAP10	Andhra Pradesh	Adilabad	Mannur	CSH9	Oct. 99–Feb. 00
NAP11	Andhra Pradesh	Adilabad	Naradegonda	CSH9	Oct. 99–Feb. 00
NAP12	Andhra Pradesh	Adilabad	Kupti	CSH9	Oct. 99–Feb. 00
NAP13	Andhra Pradesh	Adilabad	Burkapalle	JK 22	Oct. 99–Feb. 00
APAU1	Andhra Pradesh	Ranga Reddy	Rajendranagar	27B	Oct. 99–Feb. 00
APAU2	Andhra Pradesh	Ranga Reddy	Rajendranagar	296 B	Oct. 99–Feb. 00
APAU3	Andhra Pradesh	Ranga Reddy	Rajendranagar	DSV 3	Oct. 99–Feb. 00
APAU4	Andhra Pradesh	Ranga Reddy	Rajendranagar	CS 3541	Oct. 99–Feb. 00
AP0	Andhra Pradesh	Medak	Patancheru (ICRISAT)	CSH9	Oct. 99–Feb. 00
AP12	Andhra Pradesh	Mahbubnagar	Maachinenapally	Yellow Jowar	Oct. 99–Feb. 00
AP14w	Andhra Pradesh	Mahbubnagar	Maachinenapally	White Jowar	Oct. 99–Feb. 00
AP14Y	Andra Pradesh	Mahbubnagar	Maachinenapally	Yellow Jowar	Oct. 99–Feb. 00
AP15	Andhra Pradesh	Mahbubnagar	Maachinenapally	Yellow Jowar	Oct. 99–Feb. 00
AP16	Andhra Pradesh	Mahbubnagar	Maachinenapally	Yellow Jowar	Oct. 99–Feb. 00
AP16RB	Andhra Pradesh	Nalgonda	Chicholi	Yelllow Jowar	Oct. 99–Feb. 00
AP17	Andhra Pradesh	Mahbubnagar	Maachinenapally	SSG 898	Oct. 99–Feb. 00
EAP1	Andhra Pradesh	Ranga Reddy	Tukkuguda	Yellow jowar	Oct. 00–Feb. 01
EAP2	Andhra Pradesh	Ranga Reddy	Mankal	Yellow jowar	Oct. 00–Feb. 01
EAP4	Andhra Pradesh	Mahbubnagar	Kadathal	White jowar	Oct. 00–Feb. 01
EAP5	Andhra Pradesh	Mahbubnagar	Ramuntala	White jowar	Oct. 00–Feb. 01
EAP6	Andhra Pradesh	Mahbubnagar	Amanagal	White jowar	Oct. 00–Feb. 01
EAP7	Andhra Pradesh	Mahbubnagar	Velladandi	Yellow jowar	Oct. 00–Feb. 01
EAP8	Andhra Pradesh	Mahbubnagar	Machinenapally	SSG 777	Oct. 00–Feb. 01
EAP9	Andhra Pradesh	Mahbubnagar	Machinenapally	Yellow jowar	Oct. 00–Feb. 01
EAP10	Andhra Pradesh	Mahbubnagar	Machinenapally	SSG 878	Oct. 00–Feb. 01
EAP11	Andhra Pradesh	Mahbubnagar	Vangoor	Yellow jowar	Oct. 00–Feb. 01
EAP12	Andhra Pradesh	Mahbubnagar	Tandra	Yellow jowar	Oct. 00–Feb. 01
EAP13	Andhra Pradesh	Mahbubnagar	Kalwakurthy	Yellow jowar	Oct. 00–Feb. 01
EAP14	Andhra Pradesh	Mahbubnagar	Appannapalli	Yellow jowar	Oct. 00–Feb. 01
EAP15	Andhra Pradesh	Mahbubnagar	Nakkalabanda	Yellow jowar	Oct. 00–Feb. 01
EAP17	Andhra Pradesh	Mahbubnagar	Bijnapalli	White jowar	Oct. 00–Feb. 01
EAP18	Andhra Pradesh	Mahbubnagar	Palem	Improved variety	Oct. 00–Feb. 01
EAP19	Andhra Pradesh	Mahbubnagar	Ranipet	White jowar	Oct. 00–Feb. 01
EAP20	Andhra Pradesh	Mahbubnagar	Wadiyal	White jowar	Oct. 00–Feb. 01
EAP21	Andhra Pradesh	Mahbubnagar	Rayakal	Yellow jowar	Oct. 00–Feb. 01
EAP21A	Andhra Pradesh	Mahbubnagar	Rayakal	Bhoojonna	Oct. 00–Feb. 01
EAP22	Andhra Pradesh	Mahbubnagar	Palmakul	Yellow jowar	Oct. 00–Feb. 01
Cls3	Karnataka	Dharwad	Dharwad	Unknown	1997
KA53a	Karnataka	Bijapur	Almel	Fodder sorghum	Oct. 99–Feb. 00
KA58b	Karnataka	Bidar	Jalasangi	Yellow Jowar	Oct. 99–Feb. 00
KA58b-1	Karnataka	Bidar	Jalasangi	Hybrid sorghum	Oct. 99–Feb. 00
KA61	Karnataka	Bidar	ARS-Bidar	DKR 9501	Oct. 99–Feb. 00
SK1	Karnataka	Mandya	Kbettali	Popcorn	Oct. 99–Feb. 00
SK3	Karnataka	Mandya	Thendekere	local	Oct. 99–Feb. 00
SK5	Karnataka	Mandya	Laxmipura	Ogarujola	Oct. 99–Feb. 00
SK7	Karnataka	Hassan	Darsigatta	Ogarujola	Oct. 99–Feb. 00
SK12	Karnataka	Chitradurga	Hirebennur	CSH 5	Oct. 99–Feb. 00
SK13	Karnataka	Chitradurga	Obavvangathi halli	local	Oct. 99–Feb. 00
SK16	Karnataka	Chitradurga	Talaku	MSH 51	Oct. 99–Feb. 00
SK17	Karnataka	Chitradurga	Bommadevanahalli	MSH 51	Oct. 99–Feb. 00
SK-14-23	Karnataka	Shimoga	Hasalli	Ogarujola	Oct. 99–Feb. 00
SK-20-24	Karnataka	Chitradurga	Talaku	MSH 51	Oct. 99–Feb. 00
SK-22-24	Karnataka	Bellary	Amarapura	GK 52	Oct. 99–Feb. 00
UASD1	Karnataka	Dharwad	Agricultural College	CSH 13	Oct. 99–Feb. 00
Cls2	Maharashtra	Akola	Akola	Unknown	1997

(*continued on next page*)

Table 1 – (continued)

Isolate	State	District	Locality	Cultivar	Date collected
AK1	Maharashtra	Akola	Akola	Unknown	Oct. 99–Feb. 00
MH67	Maharashtra	Akola	Risode	CSH 9	Oct. 99–Feb. 00
MH70	Maharashtra	Akola	Mana	Nilva local	Oct. 99–Feb. 00
MH71	Maharashtra	Nagpur	Chembeli	Mottura	Oct. 99–Feb. 00
MH72	Maharashtra	Nagpur	Mahagaon	Kaderu local	Oct. 99–Feb. 00
MH74	Maharashtra	Yavatmal	Bori	CSH 14	Oct. 99–Feb. 00
MH75	Maharashtra	Nanded	Malegaon	Gulbhendi red	Oct. 99–Feb. 00
MH76	Maharashtra	Nanded	Limbgaon	Pandrapiwala	Oct. 99–Feb. 00
MH78	Maharashtra	Parbhani	SRS-Parbhani	CSH 9	Oct. 99–Feb. 00
MH79	Maharashtra	Parbhani	SRS-Parbhani	SPV 1333	Oct. 99–Feb. 00
NI2	Uttar Pradesh	Jhansi	Mouranipur	SBPR 94994	Oct. 99–Feb. 00
NI3	Uttar Pradesh	Jhansi	Mouranipur	ICSA 696	Oct. 99–Feb. 00
NI4	Uttar Pradesh	Nainital	Pantnagar	ICSB 91002	Oct. 99–Feb. 00
NI5	Uttar Pradesh	Nainital	Pantnagar	2219 A	Oct. 99–Feb. 00
NI6	Uttar Pradesh	Nainital	Pantnagar	2219 A	Oct. 99–Feb. 00
NI7	Uttar Pradesh	Moradabad	Droli	Local	Oct. 99–Feb. 00
NI8	Uttar Pradesh	Moradabad	Didoli/Deroli	Local	Oct. 99–Feb. 00
NI9	Uttar Pradesh	Rampur	Kunapur	Local	Oct. 99–Feb. 00
NI10	Uttar Pradesh	Moradabad	Sonali	Local	Oct. 99–Feb. 00
NI11	Rajasthan	Udaipur	RCA1	SU 1A	Oct. 99–Feb. 00
NI12	Rajasthan	Udaipur	RCA2	SU 14A	Oct. 99–Feb. 00
NI13	Rajasthan	Udaipur	RCA3	SU 27A	Oct. 99–Feb. 00
TN6	Tamilnadu	Dharmapuri	Nallgankothapalli	Yellow Jowar	Oct. 99–Feb. 00
TN10	Tamilnadu	Namakkal	Muniyappan Kovil	Solan local	Oct. 99–Feb. 00
TN11	Tamilnadu	Erode	Totivalayam	Manjal jolam local	Oct. 99–Feb. 00

[a] This study was part of a larger study that evaluated morphological and cultural characteristics of *C. africana* and *C. sorghi* in India (Muthusubramanian *et al.* 2006).

DNA extraction, DNA sequencing, and AFLP analysis

DNA was extracted from 5–10 mg lyophilized mycelium grown at 22 °C in darkness in Yeast Mold Broth (Difco, Detroit, MI) using a modified Hexadecyltrimethylammonium bromide (CTAB) DNA extraction protocol (Taylor & Powell 1982). Ribosomal DNA (rDNA) sequences were PCR-amplified and sequenced as described in Pažoutová (2001) and deposited in the EMBL database (Table 2). A corrected sequence of *Claviceps fusiformis*, differing from that used in Pažoutová (2001) was obtained. The β-tubulin (*tub2*) gene intron 3 region and elongation factor EF-1α (*tef1*) intron 4 were as described by Tooley *et al.* (2001). PCR reactions for sequencing the regions were carried out in a Perkin-Elmer Cetus model 9600 thermal cycler (Perkin–Elmer Applied Biosystems, Foster City, CA) programmed for an initial denaturation at 95 °C for 1 min, followed by 30 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 15 s, ending with a final extension at 72 °C for 6 min. Reactions contained 0.5 U Amplitaq DNA polymerase (Applied Biosystems, Foster City, CA), 1X GeneAmp PCR buffer [GeneAmp10× PCR buffer (Applied Biosystems) contains 100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01 % (w/v) gelatin], 1 μM each primer, 100 μM dNTPs, and 20 ng template DNA in a 25 μl reaction volume.

Direct-sequencing of PCR products in both directions was accomplished using an Applied Biosystems model 310 sequencer and BigDye Terminator cycle sequencing kit following purification of PCR products with either Wizard Preps (Promega, Madison, WI) or QIAquick columns (QIAGEN, Valencia, CA).

AFLPs were generated with the AFLP Analysis System II (Invitrogen, Carlsbad, CA) as previously described (Tooley *et al.* 2000). Primer sets used for analysis in the selective amplification included *MseI* + C combined with *EcoRI* + AG, *EcoRI* - AC, *EcoRI* + TA, and *EcoRI* + TT. Data were combined for the analysis. AFLP profiles were manually scored for presence or absence of bands as described previously (Tooley *et al.* 2000). Binary matrices were analysed to obtain simple matching

Table 2 – GenBank/EMBL accession numbers for DNA sequences used in this study

Species	rDNA	β-Tub	EF-1α
Epichloe amarillans	L07141	ND[a]	AF231192
E. typhina	ND[a]	X52616	AF231220
Clariceps africana	AJ011783	AF263596	AF255898
C. citrina	AJ133393	AY960840	AY960832
C. cynodontis	AJ557074	AY960846	AY960833
C. cyperi	AY387492	AY497775	ND[a]
C. fusiformis	AJ626727	AF263569	AF255891
C. gigantea	AJ133394	AY960841	AY960838
C. grohii	AJ133395	AY438671	ND[a]
C. maximensis BR	AJ133403	AY960844	AY960835
C. maximensis ZW	AJ133396	AY960845	AY960834
C. paspali	AJ133398	AF263605	AF255892
C. purpurea	AJ000069	AF263567	AF255889
C. pusilla	AJ277544	ND[a]	AY960839
C. sorghi Het	AJ849546	AY960842	AY960836
C. sorghi NAP7	AJ306621	AY960843	AY960837
C. sorghicola	AJ133397	AF263601	AF255894
C. zizaniae	AJ133405	ND[a]	ND[a]

[a] ND not determined.

coefficients among the isolates using NTSYS-pc, version 2.0 (Exeter Software, Setauken, NY). Simple matching coefficients were clustered to generate similarity trees using SAHN clustering using the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc. AFLP data were also entered into PAUP ver. 4.0b10 and analysed using NJ analysis.

Population analysis

The following measures of genetic differentiation for isolates within and among regions were calculated using Tools for Population Genetic Analysis (TFPGA) [Miller 1997: average gene diversity (H), an equivalent of the average heterozygosity for haploids (Nei 1978), proportion of polymorphic loci (Hartl & Clark 1997), and Wright's fixation index (Weir & Cockerham 1984; Wright 1978). Analysis of molecular variance (AMOVA) based on pairwise differences, and the exact test of population differentiation (Raymond & Rousset 1995) based on haplotype frequencies (both global and between pairs of populations) were performed using Arlequin 2000 (Schneider *et al.* 2000). For AMOVA, the isolates were grouped hierarchically into two groups corresponding to the two semi-arid regions of India, (one containing states Tamil Nadu, Karnataka, Andhra Pradesh and Maharashtra and the other with states Rajasthan and Uttar Pradesh; AMOVA1) and into two groups corresponding to states subdivided into districts (AMOVA2). The datasets contained all *Claviceps africana* isolates and 36 polymorphic markers.

Multilocus analysis

Random association among loci as a sign of recombination (Maynard Smith 1999) was tested by three different methods on clone-corrected datasets consisting of 58 *Claviceps africana* haplotypes and 36 polymorphic AFLP loci. All haplotypes from all states were used due to non-significant result of global and pairwise differentiation exact tests between *C. africana* populations (states). The methods were: (1) The gametic (linkage) disequilibrium test, in which the null hypothesis states that genotypes at one locus are independent from genotypes at the other locus. The program Arlequin (Schneider *et al.* 2000) runs a Fisher's exact probability test on contingency tables using a Markov chain and it was performed using the default parameters (Raymond & Rousset 1995). (2) The index of association (IA), calculated using Multilocus 1.2 (Agapow & Burt 2000). IA is a function of the rate of recombination and its value is 0 in the presence of random mating. (3) The proportion of compatible pairs of loci, calculated using Multilocus 1.2. Two loci are compatible if all the observed genotypes are explainable by mutation; no homoplasy or recombination must be inferred (Estabrook & Landrum 1975).

Phylogenetic analysis

Species and isolates used for phylogenetic analysis were as follows: *Claviceps africana* (isolate Cls-1), *C. fusiformis* (isolate Clf-1), *C. purpurea* (isolates Clp-1 and Clp-2), *C. sorghicola* (isolate Cjap-1), and *C. paspali* (isolate Cpas-1) were as described by Tooley *et al.* (2001). *C. sorghi* isolate Het6 was isolated from *Heteropogon triticeus* in India, collected at Gulbarga University campus, Gulbarga, Karnataka, India (Pažoutová *et al.* 2002). *C. pusilla* isolate Cpus-1 was isolated in 1999 in Southeast Queensland, Australia from a seedlot of *Bothriochloa* sp. *C. citrina* originated from Mexico on *Distichlis spicata* (Pažoutová *et al.* 1998). *C. grohii* (isolate 127.47) was obtained from the Centraalbureau voor Schimmelcultures (CBS). *C. gigantea* was isolated from sclerotia collected in Central Mexico (Toluca Valley). *C. maximensis* ZW2 was collected from Matopos, Zimbabwe on *Panicum maximum* by D. E. Frederickson in 2000. *C. maximensis* BR was from Brazil, on *Brachiaria brizantha* collected by E. M. Reis in 1997. *C. cynodontis* (isolate ZW2) was from Matopos, Zimbabwe from *Cynodon* sp. collected in 2001 by D. E. Frederickson. *C. zizaniae* isolate CCM 8231 was collected in Canada,1996, and isolated by L. Marvanová. The sequences of South African *C. cyperi* occurring on *Cyperus esculentus* (Naud *et al.* 2005) were deposited in GenBank by E. J. van der Linde.

Alignments were obtained using Sequence Alignment and Modelling System (SAM) based on HMM (Hidden Markov Model) (Hughey & Krogh 1996). The software is available at the server of Computational Biology Group, Computer Science and Engineering, University of California, Santa Cruz (http://www.cse.ucsc.edu/research/compbio/HMM-apps/tuneup-dna.html).

Epichloe typhina and *E. amarillans* were chosen as outgroups. The ITS sequence of *E. amarillans* originated from strain E52 (GenBank accession no. LO7141) while the EF-1α intron 4

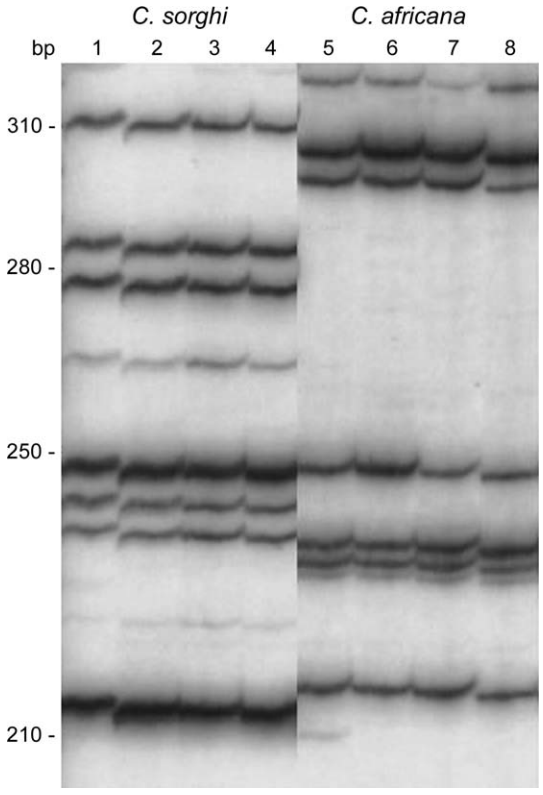

Fig 1 – Section of AFLP autoradiograph (primer EcoRI + AC) showing differences between Indian isolates of *Claviceps sorghi* and *C. africana* collected from October 1999 to February 2000. Lane 1, isolate NAP 13; lane 2 NAP5; lane 3, NAP7; lane 4, MH74; lane 5, SK5; lane 6, NI7; lane 7, NAP9; lane 8, MH78.

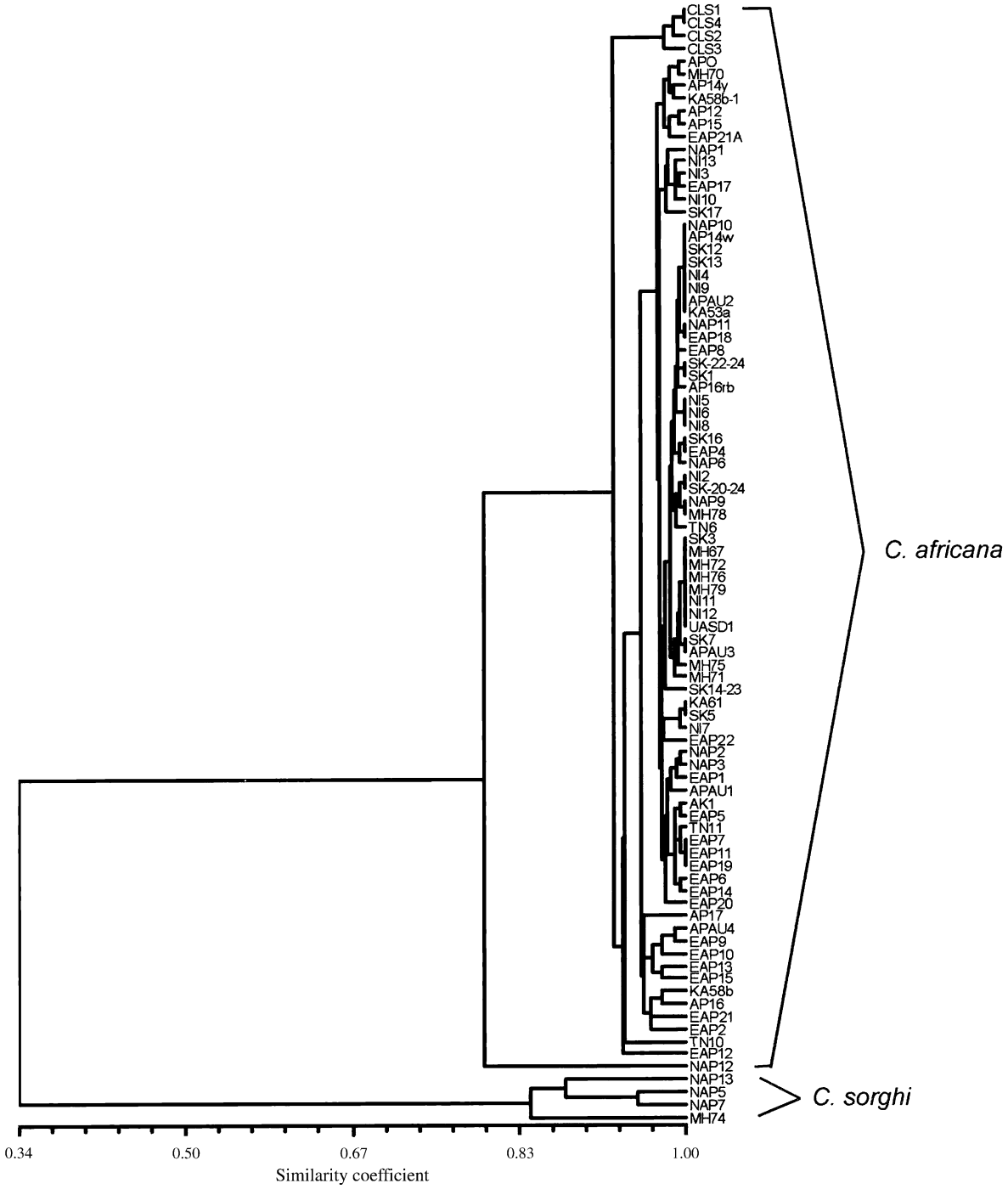

Fig 2 – Dendrogram showing similarity among isolates of *Claviceps africana* and *C. sorghi* based on AFLP analysis (data from four primer combinations combined) using simple matching coefficients. The tree was generated using the SAHN clustering program with the unweighted pair-group method with arithmetic average (UPGMA) algorithm in NTSYS-pc, version 2.0.

sequence was based on strain ATCC 200744 (AF231192). For the β-tubulin intron 3 region, the sequence of *E. typhina* strain 'PRG, isolate 85' was used (X52616) while for EF-1α intron 4, the sequence of strain ATCC200736 was used (AF231220).

The alignment was corrected manually using BioEdit version 7.0. (©1997–2004, T.Hall, Ibis Therapeutics, Carlsbad, CA). Due to the differences in mutation rate along the sequences, distance analysis was performed using Tamura–Nei (Tamura & Nei 1993) distance with the gamma model implemented in MEGA 3.0 (Kumar *et al.* 2004). Gamma distribution parameter alpha was inferred from each dataset using PUZZLE 4.0.2 (Strimmer & von Haeseler 1996) and typed into MEGA options window. The model corrects for multiple hits, taking into account the different rates of substitution between nucleotides and the inequality of nucleotide frequencies. In addition, the modified formula (Tamura & Kumar 2002) relaxing

the assumption of substitution pattern homogeneity model along the lineages was applied for *Claviceps* data. The analyses were performed with the option 'pairwise deletion' where in the computation of a distance for each pair of sequences, only the gaps involved in that particular comparison were ignored.

Results

One hundred and sixty-eight loci were scored for the four AFLP primer combinations used to analyze Indian isolates of *Claviceps africana* and *C. sorghi*; 52 loci were scored for primer set *MseI* + C/*Eco*RI + AG, 31 loci for *MseI* + C/*Eco*RI + AC, 35 loci for *MseI* + C/*Eco*RI + TA, and 50 loci for *MseI* + C/*Eco*RI + TT. Many prominent AFLP band differences were observed between *C. africana* and isolates characterized as *C. sorghi* (Fig 1). Eighty-five isolates of *C. africana* showed at or above 93 % similarity with one another, while the four isolates identified as *C. sorghi* clustered together and showed *ca* 85–95 % similarity with one another (Fig 2). *C. sorghi* as a group showed only *ca* 35 % similarity with *C. africana* (Fig 2). Results of NJ analysis in PAUP were very similar to results obtained using UPGMA analysis in NTSYS-pc so only the UPGMA results are presented.

Population diversity and distances in Claviceps africana

To investigate levels of genetic diversity present within *Claviceps africana* alone, which is currently the dominant species causing sorghum ergot in India, we compared isolates collected from the six different Indian states sampled (Tables 2 and 3).

Isolates Cls1 to Cls4, collected in 1997, grouped together with approximately 90 % similarity with the rest of the *C. africana* isolates that were collected three to four years later (Fig 2). Many isolates examined were unique in terms of their AFLP genotype (Table 3, Fig 2), but some clonal lineages were observed as well. Two major clonal groups were observed: one group consisted of isolates NAP10, AP14w, SK12, SK13, NI4, NI9, APAU2, and KA53a, while the second group consisted of isolates SK3, MH67, MH72, MH76, MH79, NI11, NI12, and UASD1 (Fig 2).

The percentage of unique AFLP genotypes for *C. africana* found in each state varied from 33 % in Rajasthan and Uttar Pradesh, to a high of 69 % in Andhra Pradesh (Table 3). The highest diversity in terms of percentage polymorphic loci (95 % criterion) was observed to occur in the state of Maharashtra, with the lowest occurring in Rajasthan. Average gene diversity (average heterozygosity) was highest in Andhra Pradesh (0.19) and lowest in Rajasthan (0.05). Nei's unbiased genetic distance estimates (Table 4) were highest between the states of Tamil Nadu and Rajasthan (0.08), and lowest between Maharashtra and Karnataka. Overall Wright's fixation index (F_{st}; Weir & Cockerham 1984) was 0.08 with 95 % confidence, intervals of 0.004 and 0.16. This means that approximately only 8 % of total gene diversity of *C. africana* in India is distributed among states.

Similarly, the AMOVA test (Table 5A) shows no divergence between the northern and central semi-arid regions. The differences between states are only 8.46 % of the total variance and most of the variance was found inside states. A parallel analysis of the data based on states and districts within India (Table 5B) has shown that most of the diversity resided at the district level. Exact tests between all pairs of states detected a significant difference only between populations of Maharashtra and Uttar Pradesh ($P = 0.01$). All other pairwise differences were not significant at the 5 % level.

Table 3 – Estimates of genetic diversity within *Claviceps africana* collections from different Indian states based on AFLP analysis[a]

State	No. of isolates	No. of unique haplotypes (%)	Percent of polymorphic loci[b]	*H*[c]
Andhra Pradesh	42	29 (69)	53.3	0.1922
Karnataka	17	5 (29)	56.7	0.1374
Maharashtra	10	5 (50)	60.0	0.1488
Rajasthan	3	1 (33)	10.0	0.0533
Tamil Nadu	3	3 (100)	33.3	0.1778
Uttar Pradesh	9	3 (33)	20.0	0.0575
Total	84	46 (55)	50.0	0.1720

[a] Data are combined from four AFLP primer pair combinations.
[b] Percentage of polymorphic loci using the 95 % criterion (Hartl & Clark 1997).
[c] Average (unbiased) gene diversity (*H*) (Nei 1978).

Gametic linkage disequilibrium in Claviceps africana

Because of low population differentiation, we considered the Indian *Claviceps africana* samples as a single interbreeding unit. Pairwise comparisons of AFLP loci among clone-corrected dataset of 58 haplotypes gave 121 out of 630 gametic disequilibrium values (19.2 %) that differed significantly from one of random mating using Fisher's exact test. The value of Index of Association was 1.32 ($P < 0.01$), significantly differing from 0. The proportion of compatible loci was 0.83 ($P < 0.01$). All of these results suggest a strong departure from a random-mating system with a dominant asexual phase and mitotic reproduction.

Low intraspecific variation in β-tubulin and EF-1α introns of Claviceps africana *and* C. sorghi

For EF-1α intron 4, sequences were nearly identical for all 85 *Claviceps africana* isolates sequenced, except for one polymorphism, where 33 isolates contained an extra GAG repeat. Within the *C. sorghi* isolates, NAP13 differed from the others in two C–T transitions. Nine *C. africana* isolates and four *C. sorghi* isolates were sequenced for the β-tubulin intron 3 region All *C. africana* isolates showed identical sequences. Within *C. sorghi*, isolate NAP5 contained an extra T.

Multigene phylogenetic analysis

Sequences of the rDNA, β-tubulin intron 3 region, and EF-1α intron 4 were obtained from several *Claviceps* species to compare gene phylogenies. In repeated attempts, we were not able

Table 4 – Nei's unbiased measures of genetic identity and genetic distance[a] for *Claviceps africana* isolates from six Indian states based on AFLP data comprising four different primer combinations

	Andhra Pradesh	Karnataka	Maharashtra	Rajasthan	Tamil Nadu	Uttar Pradesh
Andhra Pradesh	—	0.9798	0.9630	0.9366	0.9699	0.9807
Karnataka	0.0204	—	0.9935	0.9764	0.9639	0.9858
Maharashtra	0.0377	0.0065	—	0.9952	0.9509	0.9659
Rajasthan	0.0655	0.0239	0.0049	—	0.9236	0.9593
Tamil Nadu	0.0306	0.0368	0.0503	0.0795	—	0.9537
Uttar Pradesh	0.0195	0.0143	0.0347	0.0415	0.0475	—

[a] Nei's unbiased genetic identity (above diagonal) and genetic distance (below diagonal).

to amplify the EF-1α sequence of *C. grohii* nor the β-tubulin intron 3 region sequence of *C. pusilla*. The sequences of rDNA and the β-tubulin intron 3 region of *C. cyperi* (Naud *et al.*, 2005) were also included. The substitution rate was lowest for the rDNA region and highest for EF-1α (Fig 3). The divergence of EF-1α sequences posed considerable alignment problems. For example, the rDNA region of *C. pusilla* was very similar to that of *C. cynodontis* and *C. maximensis*, but its EF-1α intron 4 sequence was completely different and distant from all other *Claviceps* species studied; therefore it was omitted from the alignment.

For all three regions analysed and for the combined analysis, the three sorghum pathogens *C. africana*, *C. sorghi*, and *C. sorghicola* grouped most closely with one another and with *C. gigantea* (whose host is corn). In addition, clades of the *C. purpurea* group and the group of tropical and subtropical species were supported in all three datasets. Within the tropical group, a clade of closely related species was formed in the rDNA tree by *C. maximensis*, *C. cynodontis*, and *C. pusilla*. *C. purpurea* was found to be related to *C. grohii*, *C. cyperi*, and *C. zizaniae* in both rDNA and β-tubulin trees. *C. cyperi* and *C. zizaniae* are sister species differing from *C. purpurea* and *C. grohii* by an insertion in their ITS1 region. The phylogenetic position of the remaining species, especially that of ancestrally placed *C. paspali* and *C. citrina*, differed among single gene trees. Despite divergent levels of support for each branch among single-gene analyses, the combined dataset recovered a tree with maximum support on all branches. The single gene tree closest to the topology of the combined tree was the rDNA tree.

Discussion

Collections from ergot epidemics occurring in India in 1999–2000 and 2000–2001 (Bandyopadhyay *et al.* 2002; Navi *et al.* 2002) confirmed the presence of two types of sorghum ergot fungi that were characterized morphologically (Bandyopadhyay *et al.* 2002; Muthusubramanian *et al.* 2006). Evidence supporting the designation of some of these isolates as *Cloviceps sorghi* was reported in terms of RAPD analysis and sequence analysis of the ITS regions of ribosomal DNA (Pažoutová & Bogo 2001). Muthusubramanian *et al.* (2006) reported that of 89 isolates collected from a 1999–2000 ergot survey in India, five were *C. sorghi* and the other 84 *C. africana*. In the current study, we analysed 64 isolates from the 1999–2000 India ergot survey, 21 isolates from a 2000–2001 ergot survey in India, and four Indian isolates collected in 1997 for molecular characters. Using molecular analysis we found that four of the isolates from the 1999–2000 survey represented *C. sorghi* and the rest *C. africana*. The four isolates determined to be *C. sorghi* based on molecular characters (NAP5, NAP7, NAP13, and MH74) were confirmed as *C. sorghi* based on morphological analysis (Muthusubramanian *et al.* 2006). None of the isolates collected in the 2000–2001 epidemics represented *C. sorghi*.

A relatively low level of genetic diversity was observed within the *C. africana* populations within India sampled in 1999–2000 and 2000–2001 with similarities above 93 %. Interstate genetic distances were on the scale found previously to exist among *C. africana* populations from India, Australia, and Japan (Tooley *et al.* 2002) whereas substantially greater distances were observed in other international comparisons, for example, a genetic distance of 0.2474 between Africa and Japan

Table 5 – Analysis of molecular variance (AMOVA) table for partitioning genetic variation based on AFLP analysis of Indian *Claviceps africana* populations[a]

A

Source of variation	*d.f.*	*Sum of squares*	*Variance components*	*Percentage of variation*
Among regions	1	4.532	−0.02187	−0.80 (NS)
Among states	4	20.756	0.23031	8.46 (*)[b]
Within states	78	196.022	2.51310	92.34 (†)[b]
Total	83	221.310	2.72155	

B

Source of variation	*d.f.*	*Sum of squares*	*Variance components*	*Percentage of variation*
Among states	5	23.199	0.11865	4.31 (NS)
Among districts	19	58.248	0.24388	8.86 (*)[b]
Within districts	57	136.199	2.38946	86.83 (†)[b]
Total	81	217.646	2.75199	

NS, not significant.

[a] A, isolates were grouped hierarchically into two groups corresponding to the two semi-arid regions of India, (one containing states Tamil Nadu, Karnataka, Andhra Pradesh and Maharashtra and the other with states Rajasthan and Uttar Pradesh) and B, into two groups corresponding to states subdivided into districts. The datasets contained all *C. africana* isolates and 36 polymorphic markers.

[b] *$P < 0.001$; †$P < 0.05$.

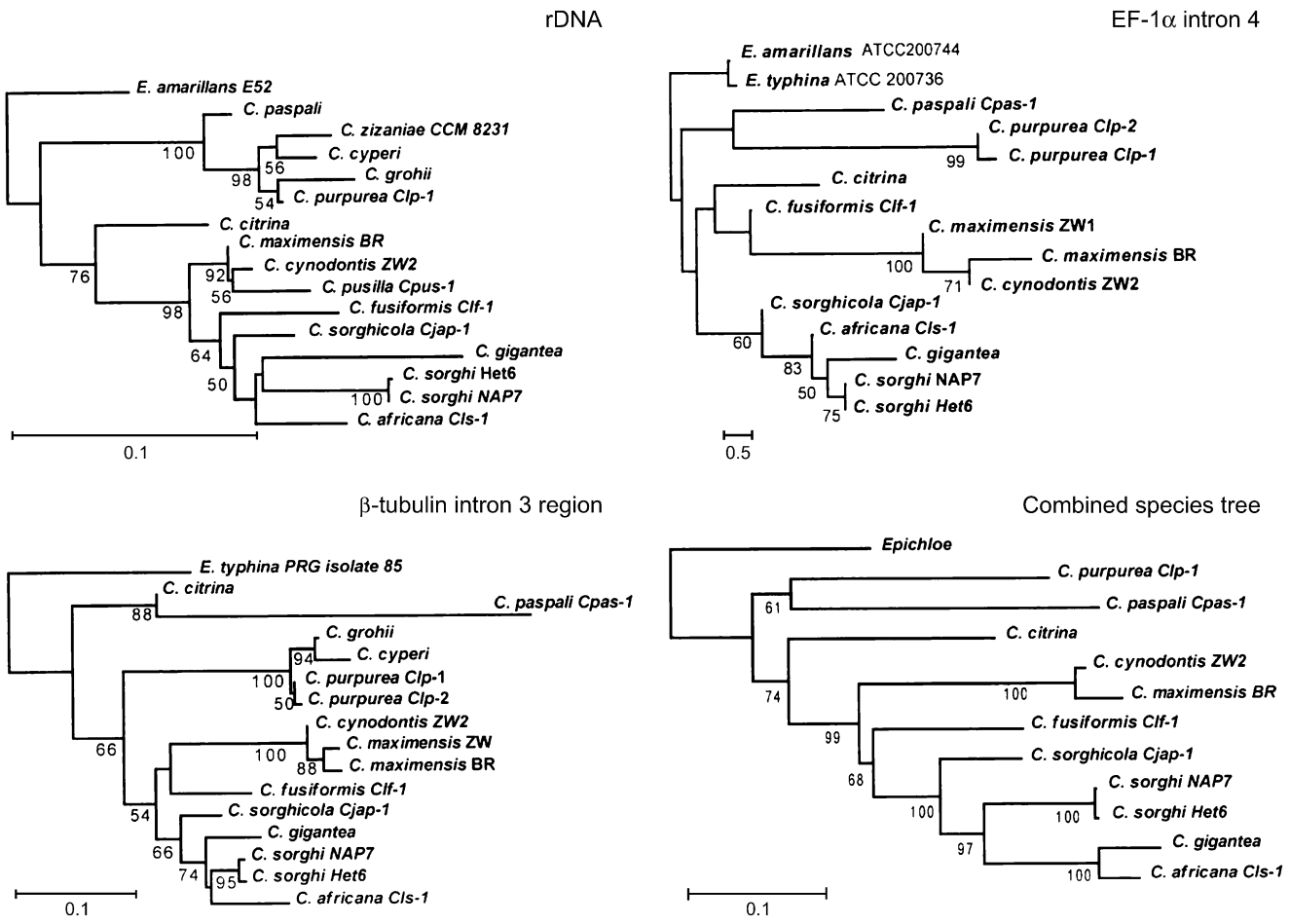

Fig 3 – Phylogenetic analysis of *Claviceps* species using rDNA, the β-tubulin intron 3 region, and elongation factor 1-α intron 4, and combined analysis from all three regions. Distance analysis was performed using Tamura–Nei (Tamura & Nei 1993) distance with the gamma model implemented in MEGA 3.0 (Kumar *et al.* 2004). Isolate designations are indicated except for isolates of *C. citrina*, *C. gigantea*, *C. grohii*, and *C. cyperi*, which were not assigned isolate numbers. *Epichloe* in the combined species tree represents combined sequence of *E. amarillans* ATCC200744 and *E. typhina* PRG isolate 85. Bar = substitutions per site.

(Tooley *et al.* 2000, Table 4). Wright's *Fst* of 0.081 calculated from the overall *C. africana* AFLP data also indicates a low level of genetic differentiation within India. Values of 0.15 and higher generally represent high genetic differentation (Wright 1978).

Isolates Cls1 to Cls4, collected in 1997, grouped together with approximately 90 % similarity with the rest of the Indian *C. africana* isolates (Fig 2). Between 1997 and the new collections made in the epidemics of 1999–2000, perhaps some genetic shift occurred resulting in the loss or gain of certain AFLP bands.

Low genetic differentiation and a departure from random allele recombination detected by three methods suggested mitotic reproduction. This correlates well with the predominating spread of *C. africana* through asexual spores, either windborne secondary conidia or honeydew-suspended primary ones. Primary conidia are protected by sugar-rich honeydew even through unfavourable periods of cold and drought, so that the fungus does not need to rely to sclerotia and perithecia formation and ascospore production for survival (Bhuiyan *et al.* 2002).

Due to the rapid spread of sorghum ergot in any given season, it is possible that one or several clones become established early in the epidemic and rise to prominence during the growing season, making it less likely that other genotypes might become established. Reservoirs in which ergot genotypes might be harboured in the absence of a crop include wild grasses such as Johnson grass (*S. halepense*). The presence of linkage disequilibrium suggests that the sexual stage of the fungus occurs in low frequency, so that novel combinations of loci are not created frequently and most of the variation results from mutation only. Sclerotia, which give rise to the sexual stage, have been shown to be very difficult to germinate in the laboratory (Frederickson *et al.* 1991) and this may be true in nature as well.

Phylogenies of *Claviceps* species resulting from rDNA and β-tubulin intron 3 region analysis were more similar than that obtained from the EF-1α gene intron 4. Moreover, this intron sequence was so divergent, that there were only few conserved sites upon which the alignment could have been 'anchored'. Intron sequences evolve rapidly and are best used for analyses among closely related species, like *Epichloe/Neotyphodium*, or for intra-species phylogenies.

Inside the genus *Claviceps*, there are groups of closely related species. In all of our analyses, the three sorghum pathogens *C. africana*, *C. sorghi*, and *C. sorghicola* grouped most closely with one another and with *C. gigantea*. As corn and sorghum are closely related hosts, these phylogenetic relationships seem to reflect host affinity.

Formerly, we assumed that *C. purpurea* (predominantly pooid hosts, Palearctic), *C. grohii* (*Carex* spp., Canada, USA) and *C. zizaniae* (*Zizania* spp., Canada, USA) (Alderman *et al.* 2004), represent ergot fungi typical of the moderate regions of the northern hemisphere. However, recent results have shown that *C. cyperi* (*Cyperus* spp., southern Africa) (Loveless 1967), is a sister species to North American *C. zizaniae*. Both species share an insertion in their ITS1 region (without any homology with GenBank database). In *C. purpurea*, *C. zizaniae*, and *C. cyperi*, the formation of peptide alkaloids was demonstrated (Kantorová *et al.* 2002; Naud *et al.* 2005) A similar situation is encountered within the tropical clade consisting of sister species *C. cynodontis* (*Cynodon* spp., *Chloridoideae*, Paleotropics), *C. maximensis* (*Urochloa maxima*, *Paniceae*, worldwide tropics). Again, although the *Claviceps* species are closely related, they colonize taxonomically very distant hosts even belonging to different subfamilies. These results suggest that there is no direct co-evolution of *Claviceps* with its hosts.

Knowledge of population genetics of Indian *C. africana* and *C. sorghi* may help shed light on possible changes to expect in other regions where sorghum ergot is newly established. Apparently, *C. africana* and *C. sorghi* can coexist side by side without detectable amounts of genetic exchange occuring. In Japan, *C. sorghicola* and *C. africana* also coexist and cause sorghum ergot, but in slightly different regions of the country. Yet, the morphology and other characteristics of the two species remains distinct (Tsukiboshi *et al.* 1999). Additional genetic and epidemiological studies of diverse populations of *Claviceps* species causing sorghum ergot in different regions of the world will help elucidate the mechanisms that govern disease occurrence and spread within this group of emerging plant pathogens.

Acknowledgements

We thank Sukumar Chakraborty and Birte Komolong for providing cultures of *Claviceps pusilla* from Australia.

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